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Pathfinding of Zebrafish CaP Motoneurons Utilizes STAT3 for Signaling from Growth Cone to Nucleus

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Summary

Neuronal pathfinding utilizes a variety of signal transduction pathways. To date experiments have revealed how these signals act locally near the activated receptors to affect growth cone dynamics. However it has not yet been demonstrated that long range retrograde signaling to the nucleus is required for pathfinding. Here I demonstrate that signaling from the transcription factor STAT3 is required for proper pathfinding of the CaP motoneuron during zebrafish development. I present evidence that STAT3 is activated by phosphorylation at the growth cone, is found along the axon, and accumulates in the nucleus of these neurons. Morpholino treatment reduces STAT3 levels and dominant negative expression as well as injection of a STAT3 morpholino or STAT3 SH2 binding peptides causes growth cones to stall. Conversely, expression of a constitutively active STAT3 induces ectopic branching and alters target choice; these neurons select an inappropriate trajectory in addition to their normal path. This is the first report of a retrograde signal emanating from the growth cone and traveling to the nucleus during neuronal pathfinding.

Introduction

Neuronal pathfinding is controlled by the activation of signal transduction pathways that modify cytoskeletal dynamics at the growth cone. Recent studies primarily on signaling by Netrin/DCC and Slit/Robo indicate that these signaling pathways are familiar pathways known for some time to regulate cell migration of

non-neural cell types especially during development [reviewed in (Deisseroth et al., 2003; Guan and Rao, 2003; Schmucker, 2003)]. This conservation of signaling pathways between the cell migration of non-neural cells and neuronal pathfinding is in some respects not surprising given the similarity of pathfinding with cell migrations. Both processes involve cytoskeletal rearrangements in response to external guidance cues. Given the conservation of signaling pathways that has emerged it is reasonable to assume that other signaling pathways required for non-neuronal cell migrations may also be required for neuronal pathfinding.

Both JAK1 kinase and STAT3 play an important role in controlling cell migrations during the development of numerous organisms from the slime mold Dictyostelium to the mouse [reviewed in (Hou et al., 2002)]. STAT3 is a transcription factor activated by cytoplasmic tyrosine phosphorylation in response to numerous growth factors and cytokines [reviewed in (O'Shea et al., 2002)]. This phosphorylation is accomplished by receptor tyrosine kinases, receptor associated kinases of the JAK family (there are four JAK kinases: JAK1-3 and Tyk2), or c-Src kinase. Phosphorylation of STAT3 leads to dimerization, movement into and retention in the nucleus, binding to specific DNA sequences, and transcriptional activation. In addition STAT3 can function cytoplasmically as an adapter allowing activation of PI3 kinase and modulation of the actin cytoskeleton (Pfeffer et al., 1997).

JAK1 kinase was first shown in the zebrafish to be required for the migration of dorsal mesendoderm during gastrulation (Conway et al., 1997). It was later shown that a downstream target of JAK1 kinase, STAT3, is also required for these cell migrations and performs the same function during mouse development (Takeda et al., 1997; Yamashita et al., 2002). It therefore is reasonable to hypothesize that JAK1 and STAT3 signaling may be required for the pathfinding of some neurons.

The possible role of STAT3 in neuronal pathfinding is bolstered by two findings. First, as reported here reducing STAT3 activity leads to a reduced escape response. Juvenile zebrafish embryos respond to gentle touch by swimming away from the stimulus. This behavior, called the escape response, prevents juvenile fish from being eaten by predators. A reduced escape response could be due to aberrant neural connections in any of the neurons that participate in this behavior or the muscles responsible for swimming. The reticulospinal neurons of the hindbrain, motoneurons in the spinal cord, and lateral muscles are required for the escape response.

The observation that interference with JAK1 or STAT3 function gives identical anterior/hindbrain defects due to early cell migration defects, yet only interference with STAT3 eliminates the escape response suggests that hindbrain/reticulospinal deficits are not responsible for the lack of this behavior. This also indicates that JAK1 kinase in this instance may not be an upstream

activator of STAT3. Rather than an early cell migration defect, STAT3 inactivation is specifically responsible for the absence of the escape response and spinal cord motoneurons or muscle defects are a likely cause. The lack of an escape response was used in a large scale zebrafish mutagenesis screen and several of these mutants have motoneuron pathfinding defects (Granato et al., 1996).

Secondly, mice lacking several CNTF family members, known activators of STAT3, have severe motoneuron deficits at birth (DeChiara et al., 1995; Forger et al., 2003; Oppenheim et al., 2001). Though pathfinding of motoneurons in these mutants was not examined, pathfinding defects could ultimately have led to the motoneuron death that was observed due to the lack of target derived survival factors. Therefore I examined motoneuron pathfinding in STAT3 deficient zebrafish embryos.

Motoneuron development in the zebrafish is quite simple compared to other organisms. Each somitic hemisegment possess three neurons called the RoP (rostral primary), MiP (middle primary), and CaP (caudal primary) that project to the somitic muscles (Eisen, 1991; Eisen, 1999). The soma of these neurons lie next to each other in a rostral to caudal position within the ventral spinal cord (Figure 2A). These neurons are easily identified based on soma size, shape, and position. The rostral to caudal soma position correlates with the final muscle target that is innervated.

All three neurons extend axons that fasciculate and project to a position called the choice point between the dorsal and ventral muscle groups. The axon of the RoP projects no further, while the MiP axon sends a collateral projecting to the dorsal aspect of the embryo (the dorsal target) and eventually wraps around the dorsal muscle group. The CaP axon continues its ventral path to the ventral target and eventually wraps around the ventral muscle group. Half of all hemisegements possess an additional neuron, the VaP (variable primary). The soma of this neuron lies caudal to the CaP soma and projects an axon to the choice point. This neuron dies between the first and second day of development (Eisen et al., 1990). In addition to the four primary motoneurons (RoP, MiP, CaP, and VaP) an additional group of motoneurons (secondaries) project axons later in development along paths similar to the primaries. In this paper I will only address the role of STAT3 in the primary motoneurons.

I have found that in agreement with the reduced escape response, STAT3 knockdown embryos produced by injection of a STAT3 morpholino (MO) have primary motoneuron pathfinding defects while JAK1 knockdown embryos have normal motoneuron projections. Expression of a dominant negative STAT3 or injection of peptides that block STAT3-STAT3 dimerization also lead to pathfinding defects. Specifically the ventral projection of the CaP motoneuron either does not occur after the choice point or is stalled along the ventral path. Interestingly, expression of a constitutively active version of STAT3 leads to the

opposite effect of inactivation resulting in ectopic axonal branching and promiscuous target choice.

To verify that activated STAT3 exists in motoneurons, embryos were stained with a phosphospecific STAT3 antibody. Activated STAT3 was found in the soma of CaP neurons that have reached the choice point or have projected ventrally just beyond it. Closer examination of anti-phospho-STAT3 staining reveals phosphorylated STAT3 at growth cones and along axons.

To date our understanding of downstream pathways required for neuronal pathfinding has led us only a short distance from the activated receptors. The findings presented here indicate that in addition to these local signals retrograde signaling to the nucleus is also required, at least for the CaP motoneuron. Certainly for synaptogenesis and more generally for learning and memory, retrograde signaling is required [reviewed in (Deisseroth et al., 2003; Kalinovsky and Scheiffele, 2004; Kandel, 2001)]. These retrograde signals enter the nucleus and subsequent transcriptional events ultimately modulate synaptic connections. Similarly during pathfinding retrograde signals leading to transcriptional activation modulate growth cone dynamics and pathfinding choice. Here I present the first report of long-range retrograde signaling during neuronal pathfinding.

Results

Reduced STAT3 and JAK1 Signaling Lead to Similar Morphological Defects but Dissimilar Escape Responses

Both JAK1 and STAT3 are required for early cell migrations during zebrafish gastrulation (Conway et al., 1997; Yamashita et al., 2002). As shown in figure 1 eliminating the activity of either JAK1 or STAT3 results in nearly identical morphologies. Inactivation of STAT3 (Figure1B and 1G) and JAK1 (Figure 1D and 1I) by morpholino injection both lead to a reduction in anterior structures rostral to the otocyst, readily apparent in the 27 hour post-fertilization (p.f.) embryos shown in figure 1. JAK1 is also required for posterior somite formation with JAK1 inactivation leading to a slight spadetail phenotype (Figure 1D, arrow). Proof that the morpholinos specifically reduce the activity of their cognate genes is shown by injection of dominant negative versions that result in defects identical to those seen upon morpholino injection (Figure 1C and H for STAT3, E and J for JAK1).

The dominant negative STAT3 was constructed by mutating tyrosine 708 to phenylalanine (called $STAT^{YF}$-GFP) in the activation loop of STAT3. STAT3 is activated by phosphorylation of tyrosine 708 on a flexible loop that can reach across to the SH2 domain of another STAT monomer. Mutual SH2 interactions lead to dimerization. $STAT3^{YF}$ can still bind to receptor docking sites and when over expressed will prevent endogenous STAT3 from associating with its cognate receptor and undergoing tyrosine phosphorylation, dimerization, and

transcriptional activation. In this manner $STAT3^{YF}$ acts as a dominant negative. STAT3 can form homodimers or heterodimers with STAT1, however, heterodimer formation in the early zebrafish embryo can be ruled out because STAT1 is not expressed prior to 6 days p.f. (Oates et al., 1999).

In experiments detailed later it was important to tag STAT3 constructs with GFP so in all experiments reported here STAT3 and JAK1 were expressed as carboxyterminal GFP fusions. The JAK1 dominant negative replaced lysine 717 for glutamic acid. This lysine is required for JAK1 kinase activity (Conway et al., 1997). Both dominant negative STAT3 (Figure 1C and H) and dominant negative JAK1 (Figure 1E and J) lead to identical anterior deficits. Both the morpholino and dominant negative defects can be rescued by injection of RNA encoding the cognate wild-type proteins [not shown and (Conway et al., 1997)].

The ability of GFP tagged STAT3 to rescue the cell migration defect as well as the ability of the STAT3-GFP fusion protein to rescue another defect, neuronal pathfinding (detailed below), shows that carboxyterminal GFP fusions with STAT3 are functionally active. Numerous studies in addition to the ones reported here have used carboxyterminal GFP fusions of STAT3 and in all studies fusion with GFP has not altered STAT3 function (Kretzschmar et al., 2004; Pranada et al., 2004).

Despite similar morphology, embryos with reduced STAT3 and JAK1 signaling have different escape responses. In response to touch juvenile zebrafish quickly swim away from the stimulus. Uninjected embryos and embryos injected with JAK1 morpholino or dominant negative JAK1 display a robust escape response at two days of development. In contrast, embryos where STAT3 signaling has been eliminated by either morpholino or dominant negative injection have a reduced escape response. These embryos are either non-responsive to touch or require multiple proddings to elicit swimming.

Morpholino Knockdown of STAT3 Results in Pathfinding Defects

The escape response is a hardwired behavior that utilizes neurons of the hindbrain, spinal motoneurons and somitic muscles. A reduced escape response in STAT3 deficient embryos suggests defects in one or more of the elements required for this behavior. The finding that both JAK1 and STAT3 deficient embryos have identical hindbrain deficits while only STAT3 deficient embryos have a reduced or absent escape response suggest defects either in motoneurons or somitic muscles. Because an antibody exists that allows easy visualization of motoneurons I began examining the cause of the reduced escape response with an examination of these neurons.

Pathfinding of motoneurons occurs in a temporal rostral to caudal fashion; rostral neurons project axons before caudal neurons. I examined 27 hour p.f. embryos because at this time roughly half of the primary motoneurons have

reached the ventral target while the remainder are still in the process of pathfinding. By examining motoneurons in different rostral to caudal somites in embryos at this stage one can examine the entire pathfinding process from emergence of axons to arrival at the dorsal and ventral targets in a single individual. Also, at this stage the axons of secondary motoneurons have not yet emerged from the spinal cord, so one can exclusively examine primary motoneuron pathfinding. Motoneurons were examined using the ZNP1 antibody that recognizes both primary and secondary motoneurons. Unfortunately no antibody exists that only recognizes primary motoneurons.

Uninjected (Figure 2B) and JAK1 MO injected (data not shown) embryos show normal dorsal and ventral motoneuron projections. On the other hand, pathfinding of CaP motoneurons in STAT3 MO injected embryos is delayed while MiP dorsal projections (Figure 2C, white arrowheads) are normal. Because the RoP axon fasciculates with the MiP and CaP axons and is always associated with them it is not possible to assess pathfinding defects in this neuron by antibody staining alone.

To quantitate pathfinding defects, CaP axons were classified into one of three categories: (type 1 neurons) those having projected to the ventral target, (type 2 neurons) those having projected past the choice point but not having arrived at the ventral target, and (type 3 neurons) those not having projected past the choice point (Figure 2E). Though embryos within a clutch are remarkably

synchronous in their development, to facilitate the comparison of individuals to each other only embryos were compared that possessed 22 motoneurons per side that had at least reached the choice point. In uninjected embryos, type 2 and 3 neurons are confined to caudal somites (Figure 2F). In STAT3 knockdown embryos (Figure 2G) CaP motoneurons often fail to project past the choice point or stall shortly after projecting along the ventral pathway; type 2 and 3 neurons are found in rostral as well as caudal somites.

In a second experiment, I determined if pathfinding defects resulting from STAT3 MO injection could be rescued by injection of wild-type $STAT3^{WT}$-GFP encoding RNA. This RNA possessed the coding region of STAT3-GFP fused behind the 5' untranslated region of the β-globin gene. The STAT3 MO targets the endogenous 5' UTR sequences in STAT3, so the MO is unable to inactivate the injected RNA. In MO plus RNA injected embryos GFP fluorescence was seen confirming expression of $STAT3^{WT}$-GFP and the inability of the MO to inactivate the injected RNA. Injection of this RNA rescued pathfinding defects (Figure 2I) just as it was also able to rescue cell migration defects shown in figure 1 (not shown). Over expression of wild type STAT3 has no affect upon pathfinding (Figure 2D and H).

Expression of a Dominant Negative STAT3 and Injection of STAT3 SH2 binding Peptides Confirm a STAT3 requirement for Neuronal Pathfinding

To further confirm that pathfinding defects are specifically due to inactivation of STAT3, two experiments were performed. First, RNA encoding the dominant negative version of STAT3 ($STAT3^{YF}$-GFP) was injected into one-cell embryos and motoneurons examined. If CaP pathfinding defects are specifically due to inactivation of STAT3, then the dominant negative should recapitulate the STAT3 MO effect as it does for early cell migration defects shown in figure 1. Indeed, $STAT3^{YF}$-GFP expression causes CaP pathfinding defects (Figure 3A). This effect is not as pronounced as MO injection; the majority of defects are slowed (type 2) rather than stalled (type3) axons (Figure 3E).

In a second experiment, peptides were injected into one-cell embryos that will disrupt STAT3-receptor and STAT3-STAT3 interactions. If STAT3 is required for CaP pathfinding then disruption of these specific interactions should lead to pathfinding defects. These peptides comprised either the activation loop of STAT3 possessing a phospho-tyrosine or a mutant version of this sequence used as a control. The SH2 domain of STAT3 specifically binds the activation loop sequence and only if tyrosine 708 in this sequence is phosphorylated. As a control, a peptide consisting of the activation loop was synthesized, however tyrosine 708 was mutated to a phenylalanine. This change is analogous to the mutation produced in the dominant negative version of $STAT3^{YF}$. In both instances the tyrosine that undergoes phosphorylation was mutated to a structurally similar amino acid, however one that can not undergo phosphorylation and hence does not possess a negative charge required by all

SH2 domains for binding. The control peptide tests if injection of foreign peptides affects development and specifically neuronal pathfinding.

One caveat to the use of the phospho-tyrosine peptide is the potential loss of the phosphate group due to phosphatases. Because these peptides are injected at the one-cell stage the phospho-tyrosine peptide must remain in the phosphorylated state for at least 17 hours within the embryo till motoneuron pathfinding commences. Thus a negative result with the phospho-tyrosine peptide does not necessarily indicate that STAT3 is not required for pathfinding. To circumvent this potential problem an additional peptide was synthesized containing a non-hydrolysable analogue of phospho-tyrosine. This peptide is identical to the control and phospho-tyrosine peptides except for possessing phosphonomethylene-phenylalanine. This amino acid is structurally similar to phospho-tyrosine, possessing the negative charge of a phosphate group while remaining resistant to hydrolysis (Burke et al., 1994).

The control peptide has no affect upon motoneuron pathfinding (Figure 3B). When pathfinding is correlated with somite position type 2 and type 3 CaP motoneurons are found only in caudal segments, identical to that found for uninjected embryos (Figure 3F, compare with Figure 2F). The phospho-tyrosine version of this peptide on the other hand causes CaP motoneurons to stall either at the choice point or shortly beyond it (Figure3C and G). It would appear that

phosphatases do not dephosphorylate the tyrosine residue in this peptide or this peptide if dephosphorylated can undergo rephosphorylation.

Note that the only difference between the control peptide and the phospho-tyrosine peptide is the presence of a phosphate group. The requirement for the phosphate group suggests that a specific interaction requires a negative charge in this sequence context. This strongly implicates STAT3 since the SH2 domain of STAT3 specifically binds this sequence and only in the phosphorylated state.

As expected the phosphonomethylene-Phe peptide also inhibits CaP pathfinding (Figure 3D and H). The peptides were injected at three concentrations, pathfinding quantitated, and compared to the effect of STAT3 MO injection or expression of the dominant negative STAT3^{YF}-GFP (Figure 3I). This quantitation was accomplished by summing the pathfinding failures between somites 8 to 17 inclusive. For uninjected embryos nearly all CaP neurons in these somitic segments have reached the ventral target. This approach gives a bioassay allowing quantitative comparison of the ability of a peptide or treatment to inhibit neuronal pathfinding.

Two points are interesting from this quantitative analysis. First, no condition yields more than a 50% inhibition of pathfinding suggesting that although STAT3 participates in pathfinding it is not obligatorily required. Second,

the phospho-tyrosine peptide inhibited pathfinding equally over a 100 fold dilution, however, a dose response is seen with the phosphonomethylene-Phe containing peptide. The phospho-tyrosine containing peptide gives maximal inhibition while the analogue containing peptide is not as active at inhibiting pathfinding and presumable not as active at preventing STAT3 phosphorylation and dimer formation. In agreement with this finding is the report that a phosphonomethylene-phenylalanine containing peptide used to inhibit the activity of PI3 kinase possesses a 5 fold lower binding constant for the SH2 domain of this protein than the corresponding phospho-tyrosine containing peptide (Burke et al., 1994). In general both the phospho-tyrosine and phosphonomethylene-Phe containing peptides are potent inhibitors of neuronal pathfinding. The concentration for half maximal inhibition of CaP pathfinding (IC_{50}) for the phospho-tyrosine peptide is <35nM intracellular concentration while the IC_{50} for the phosphonomethylene-Phe peptide is between 350nM and 35 nM. Both the phospho-tyrosine and phosphonomethylene-Phe containing peptides also inhibited gastrulation movements resulting in reduced head structures (not shown).

Constitutively Active STAT3 leads to Ectopic Branching

The finding that STAT3 is required for CaP pathfinding raises the obvious question: will activated STAT3 enhance pathfinding? To address this question a constitutively active version of STAT3 was constructed by mutating two amino acid residues to cysteines. Darnell and colleagues have shown that conversion

of two amino acids (for zebrafish STAT3 alanine 663 and asparginine 665) to cysteine leads to a disulfide bridged dimer (Bromberg et al., 1999). Crystal structure has shown that in the STAT3 dimer these residues of each monomer are closely opposed (Becker et al., 1998; Chen et al., 1998). Substitution of cysteine for these residues leads to a permanent dimer, constitutive transcriptional activation of genes, nuclear localization, and transformation of cells (Bromberg et al., 1999).

Expression of constitutively active STAT3 ($STAT3^{AC/NC}$-GFP) leads to an opposite effect of inhibiting STAT3, causing ectopic branching of CaP motoneurons (Figure 4B, red arrow heads). To quantitate this effect, the number and length of branches for CaP motoneurons in somites 8 through 12 inclusive were measured. The CaP axons of only these somites were counted because the CaP projections of somites 1 through 7 (rostral somites) are already more branched than those in other somites making measurements difficult. Only branches longer that 4 microns were included in my analysis, since varicosities in the main axonal trunk of CaP motoneurons (somites 8-12 inclusive) can be as wide as 3 microns. Also, only branches ventral to the choice point were counted, since branches more dorsal to this point could originate from RoP or MiP neurons. In uninjected embryos, CaP motoneurons possess on average 2 branches ventral to the choice point and expression of wild-type STAT3 has no affect upon the number of branches (Figure 4C). Expression of constitutively active STAT3 increased this number to an average of 8 branches. Though the

number of branches was increased, the length of the branches was unchanged (10μ ± 6μ for uninjected, 10μ ± 5μ for STAT3^{WT}-GFP injected, 8μ ± 3μ for STAT3$^{AC/NC}$-GFP injected). In no instance did branches migrate over somite boundaries. Though STAT3$^{AC/NC}$-GFP increased the number of branches, constitutively active STAT3$^{AC/NC}$-GFP did not accelerate pathfinding. The number of CaP neurons that reached the ventral target, the choice point or an intermediate position was unchanged (Figure 4D).

STAT3 is Activated in CaP Motoneurons

Inhibition of CaP pathfinding by a STAT3 MO, expression of a dominant negative, and injection of STAT3 SH2 binding peptides suggests that activated STAT3 exists in these neurons or the muscle targets. Embryos were probed with a phospho-specific STAT3 antibody to identify the location of phosphorylated STAT3. This antibody has been shown to specifically interact with the phosphorylated form of zebrafish STAT3 (Yamashita et al., 2002). Phosphorylated STAT3 is found in motoneurons and the size, shape and position of stained soma indicates that they are CaP neurons (Figure 5A-D). Staining was not found in muscles or the RoP or MiP primary motoneurons. Phosphorylated STAT3 is also found in isolated EVL cells that are seen out-of-focus in figure 4A. These cells form a “skin” that covers the embryo and is sloughed-off later in development. Phosphorylated STAT3 is found in caudal neurons that are at or have projected just past the choice point. Interestingly, rostral CaP neurons also possess phosphorylated STAT3 (Figure 5A and E).

STAT3 is Activated at Growth Cones and is found along Axons

A closer examination of phospho-STAT3 staining reveals that phosphorylated STAT3 is present at growth cones of CaP motoneurons (Figure 6). Rostral CaPs possess more branches than CaP axons in trunk or caudal somites and the tips of these branches possess phosphorylated STAT3 (Figure 6A-I). In caudal somites phosphorylated STAT3 is found at growth cones that have either just arrived at the choice point or progressed just past it (Figure 6J-O). Staining could also be found along axons leading to the neuronal soma (Figure 6P-R). In no case was staining found at growth cones or along axons where the soma was not also stained. The phospho-STAT3 stained soma are not shown in panels A-I.

STAT3 MO injection reduces Activated STAT3 in Embryos

The finding that pathfinding defects can be rescued by the injection of RNA encoding wild-type STAT3 indicates that the MO specifically knocks down STAT3 function. To further confirm that the MO reduces STAT3 signaling, embryos injected with the MO were stained with the phospho-specific STAT3 antibody (Figure 7D-F) and compared to uninjected embryos (Figure 7A-C). As expected, the STAT3 MO eliminates almost all staining, though a few EVL cells continue to possess phosphorylated STAT3.

STAT3 is Required Cell Autonomously for Pathfinding

The observation that activated STAT3 is found in CaP neurons and not in muscle targets suggests that STAT3 is required cell autonomously in CaP neurons for pathfinding. To confirm a cell autonomous requirement for pathfinding, cell transplants were performed where wild-type blastomeres from rhodamine injected embryos were transferred into unlabeled STAT3 MO-injected hosts (Figure 8D-F). By placing the transplanted cells in the region of the gastrula fate map that is destined to produce spinal cord neurons one can increase the chance that the transplanted cells develop into these cells rather than the muscle targets.

To confirm that the transplant technique does not have deleterious affects upon the transplanted cells a control was performed were wild-type cells were transplanted into wild-type hosts (Figure 8A-C). In both the wild-type to MO and the control transplants all of the wild-type blastomeres that developed into CaP neurons underwent proper pathfinding (Figure 8). Any embryos where transplanted cells developed into somitic muscle were discarded.

In total, transplanted wild-type cells developed into 54 CaP motoneurons in 15 MO hosts and into 28 CaP motoneurons in 24 wild-type hosts in somites 8 to 17 inclusive. Only transplanted cells that developed into CaP neurons in these somites were counted. The reason for restricting quantitation to these somite positions is because CaP neurons in more caudal positions even of wild-type embryos have failed to pathfind to the ventral target at the developmental stage

examined (27 hours p.f.), while somites in more rostral positions, even in MO injected embryos, have a low probability of pathfinding failure. The CaP neurons of MO injected embryos between somites 8 to 17 inclusive have on average a 38% failure rate (Figure 3I). Thus if STAT3 is required non-cell autonomously one would have expected to find at least 20 transplanted CaPs with pathfinding failure among the 54 neurons examined in MO hosts. All 54 transplanted neurons underwent normal pathfinding.

Late Expression of Constitutively Active STAT3 Leads to Promiscuous Pathfinding

As shown above, expression of constitutively active STAT3$^{AC/NC}$-GFP leads to ectopic branching if expressed in the CaP motoneuron throughout its axonal pathfinding. This expression was accomplished by injecting RNA encoding activated STAT3 into one-cell embryos. However, will constitutively activate STAT3$^{AC/NC}$ induce ectopic branching or alter morphology if expressed in CaP motoneurons after pathfinding?

To address this question I expressed STAT3$^{AC/NC}$-GFP under the control of a neuro-specific promoter that becomes active late in motoneuron pathfinding. I used a transient expression assay rather than transgenics for this analysis. Zebrafish embryos injected with a DNA construct containing a tissue specific promoter show mosaic expression of the transgene, rather than transcription throughout the tissue. For the analysis of gene function in neurons mosaic

expression is advantageous. If the transgene is linked to GFP, expressing neurons can be identified from non-expressing neurons and the detailed morphology of these neurons can be viewed upon a dark background of non-expressing cells.

The promoter I used is the GATA2 minimal promoter containing a neuro-enhancer, a promoter expressed in all neurons and occasionally EVL cells (Meng et al., 1997). Expression from this promoter begins at 24 hours p.f. in motoneurons that possess axons along the ventral pathway or that have reached the ventral target. Expression peaks at 48 hours remaining constant for several days. The embryos I injected were examined at 48 hours p.f.

As expected, expression of GFP or $STAT3^{WT}$-GFP under GATA2 control (called GATA2:GFP and GATA2:$STAT3^{WT}$-GFP) has no effect upon branching of any of the three primary motoneuron types (Figure 9A-C, RoP and MiP images not shown, table 1). Also, expression of GATA2:$STAT3^{AC/NC}$-GFP in RoP or MiP neurons has not effect upon their morphology (images not shown, table 1). However fifty percent of CaP neurons expressing GATA2:$STAT3^{AC/NC}$-GFP possess an abnormal collateral that projects dorsally at the same position as the dorsal collateral of the MiP axon (Figure 9E-M, white arrows in F, I, and L, table 1). These neurons chose both the dorsal and ventral pathways; they were promiscuous. Although this collateral initially follows the path of the MiP axon it usually fails to follow the dorsal pathway; rather it projects caudally following the

neuropil of the spinal cord (Figure 9K-M). Blue arrows in figure 9 mark EVL cells that occasionally show expression from this promoter construct.

Unlike previous experiments where the axons of secondary motoneurons have not yet exited the spinal cord, in these experiments using 48 hour p.f. embryos secondary motoneurons are pathfinding along the dorsal and ventral pathways. Though the size, shape and position of the soma of bifurcated neurons indicated they are primary CaP motoneurons, I wanted to further confirm they were primary neurons.

GATA2:STAT3$^{AC/NC}$-GFP expressing embryos were stained with the ZN5 monoclonal antibody. This antibody specifically labels secondary motoneurons, rather than both primaries and secondaries like the ZNP1 antibody. Primary and secondary motoneurons do not always follow the same exact paths. Because of this I was able to identify bifurcating neurons that failed to stain with ZN5 confirming that they are primary CaP neurons (Figure 9O-R). Notice in figure 9R that ZN5 and GFP staining are not coincident. It should be noted that in these experiments some secondary neurons expressed GATA2:STAT3$^{AC/NC}$-GFP (not shown). These neurons could be unambiguously identified as secondaries based on their soma placement far from the spinal cord exit point as well as their soma size, ventral soma position within the spinal cord, and ZN5 staining. Secondary neurons also bifurcated when expressing GATA2:STAT3$^{AC/NC}$-GFP.

This is interesting given that secondary motoneurons do not possess phosphorylated STAT3 even up to 72 hours p.f. (data not shown).

Discussion

Here I have shown that STAT3 is required for neuronal pathfinding of primary CaP motoneurons in the zebrafish. Morpholino knockdown, expression of a dominant negative $STAT3^{YF}$, and injection of STAT3 SH2 binding peptides lead to pathfinding defects. Morpholino defects can be rescued by expression of wild-type STAT3 and these defects are cell autonomous as shown by cell transplantations. While elimination of STAT3 signaling leads to pathfinding defects the reverse effect is seen when constitutively active $STAT3^{AC/NC}$ is expressed either in the whole embryo or specifically in the CaP motoneuron. Constitutive activation of STAT3 results in ectopic branching and promiscuous target selection. These findings add neuronal pathfinding to the growing list of functions performed by STAT3.

The requirement for STAT3 in pathfinding raises the question: what signaling pathways activate STAT3 in CaP neurons, how are STAT3 signals transported to the nucleus, and what downstream targets are required for pathfinding? To approach these questions it is useful to place STAT3 in the context of known signaling mechanisms and specifically those activated by guidance cues.

STAT3 Activation

Signaling by STAT3 and its function in neuronal pathfinding can be divided into several steps (Figure 10). At or near the choice point a factor must activate the phosphorylation of STAT3 (Figure 10, Activation). A neurotrophic candidate that could attract CaP motoneurons as well as activate STAT3 is HGF/scatter factor. The receptor for HGF/scatter factor, c-Met, is known to activate STAT3 (Boccaccio et al., 1998) and HGF/scatter factor has been found to be a neurotrophic factor for limb innervating motoneurons [(Ebens et al., 1996) reviewed in (Birchmeier and Gherardi, 1998)]. A finding that supports a role for HGF/scatter-factor in CaP pathfinding is the presence of c-Met in these neurons (Segawa et al., 2001). This expression occurs at the time of pathfinding and neither the RoP nor MiP expresses c-Met. Also, HGF/scatter factor binds to extracellular matrix components and could be tethered to the medial surface of the somitic muscles. One would expect the neurotrophic factor for CaP motoneurons to be anchored closely to the muscle surface.

Other possible neurotrophic factors are members of the CNTF family. Members of this group of neurotrophic factors (CNTF, CLC, CLC-CLF dimer, CT, NP) activate STAT3 (Derouet et al., 2004; Lesser and Lo, 2000). These proteins are unable to activate their receptors by themselves but require dimerization with an accessory binding factor or ligand called the CNTFα-receptor (Davis et al., 1993a; Davis et al., 1993b; Davis et al., 1991; Derouet et al., 2004; Lesser and

Lo, 2000). Though called a receptor, CNTFα is actually a co-ligand, only when bound to CNTFα can these CNTF members activate their receptors (Elson et al., 2000; Ip et al., 1993; Lelievre et al., 2001; Plun-Favreau et al., 2001).

Mouse knockouts of the CNTFα receptor, CLF, and CT display a pronounced reduction in the number of motoneurons at birth (DeChiara et al., 1995; Forger et al., 2003; Oppenheim et al., 2001). Though the precise reason for the reduction of motoneurons in these knockouts is not known, one explanation would be an initial defect in pathfinding that would prevent exposure to target derived factors required for neuronal survival. NP, CT, CLC, CLF, and the CNTFα receptor are highly expressed by mouse embryonic skeletal muscle (Derouet et al., 2004; Forger et al., 2003; Helgren et al., 1994; Pennica et al., 1996). Perhaps they are similarly expressed by somitic muscles of the developing zebrafish. Thus these proteins are good neurotrophic candidates for CaP motoneurons. The CNTFα receptor is GPI linked and would be closely associated with the muscle surface.

Local Signaling by STAT3 and Local Signaling to STAT3

STAT3 possesses a mechanism for locally modulating the actin cytoskeleton (Figure 10, Local Signaling). STAT3 can act as an adapter protein, linking the regulator subunit of PI3 kinase (p85α) to receptors (Pfeffer et al., 1997). STAT3 possesses a p85α binding site, that when tyrosine phosphorylated (Y658 in zebrafish STAT3) binds to the SH2 domain of p85α. Once bound to STAT3,

p85α is itself tyrosine phosphorylated and thereby activated. p85α can then modulate the actin cytoskeleton either directly through cdc42 (Jimenez et al., 2000) or through activation of PI3 kinase (p100) [reviewed in (Merlot and Firtel, 2003)].

The role of PI3 kinase and associated downstream transduction pathways in cell motility is well established [reviewed in (Merlot and Firtel, 2003)]. Like JAK1 and STAT3, PI3 kinase is required for cell migration of gastrulating mesendodermal cells in the zebrafish (Montero et al., 2003). PI3 kinase has also been shown to regulate neurite extension of neurons in culture (Kimura et al., 1994; Kita et al., 1998; Kobayashi et al., 1997; Korhonen et al., 1999; Posern et al., 2000; Sanchez et al., 2001). The adapter function of STAT3 bridges neurotrophic stimulation to PI3 kinase pathways that in turn can regulate local actin cytoskeletal dynamics. Activation of PI3 kinase pathways can also lead to STAT3 phosphorylation via Rho GTPases (Aznar et al., 2001; Pelletier et al., 2003; Ram et al., 2000; Simon et al., 2000). Thus there is reciprocal signaling between STAT3 and PI3 kinase pathways that link cytoskeletal modulation and transcriptional activation events.

Part of the pathfinding defects I observe upon MO knockdown of STAT3 could therefore be due to perturbation of local signaling. Elimination of STAT3 could reduce p85α and PI3 kinase activation. However, pathfinding defects observed by expression of the dominant negative $STAT3^{YF}$-GFP are unlikely to

be due to local signaling defects. Though $STAT3^{YF}$-GFP can not be phosphorylated on Y708 and therefore act as a transcription factor, it should still bind via its SH2 domain to phosphorylated receptors and undergo Y658 phosphorylation. Thus $STAT3^{YF}$ would still possess adapter function and lead to p85α and PI3 kinase activation. A rigorous examination of the role of p85α and p110 in STAT3 mediated pathfinding will require expression of a Y658 STAT3 mutant.

Axonal Translocation of STAT3 Signals

In non-neuronal cells, translocation of activated STATs from the cell membrane to the nucleus is a short journey and presumably occurs by diffusion. My finding that STAT activation occurs at growth cones sometimes over 50 microns from the nucleus suggests that a specific retrograde axonal transport mechanism may be required. Two mechanisms for retrograde signal transport are known (Figure 10, Retrograde Transport).

In one mechanism signals are translocated along axons by signaling endosomes [(Delcroix et al., 2003; Howe and Mobley, 2004; Ye et al., 2003)]. In this case the entire receptor and attached ligand are endocytosed (Figure 10, Signaling Endosome). As the vesicle travels, continual ligand stimulation of the receptor produces activated members of transduction cascades along the axon. If STAT3 signals through endosomes, the STAT3 proteins phosphorylated at the CaP growth cone may not be the same proteins that enter the nucleus.

The second mechanism for signal translocation (Figure 10, Axoplasmic Importin) is through axoplasmic importins (Hanz et al., 2003). Importins, also known as karyopherins, are factors that bind proteins and mediate their translocation into the nucleus. It is possible that the karyopherin that binds STAT3 dimers, which to date is unknown, may reside in CaP axons and growth cones and by a dynein mediated mechanism translocate STAT3 to the cell body.

Transcriptional Activation by STAT3

As mentioned above STAT3^{YF}-GFP may still be able to affect local signaling, however it can not undergo the dimerization that is essential for its function as a transcription factor. The observation that STAT3^{YF}-GFP causes pathfinding defects indicates that transcriptional activation is required for CaP pathfinding (Figure 10, Transcriptional Activation). In CaP motoneurons STAT3 activation is transient and presumably a brief transcriptional event is required.

A systematic search for genes activated by STAT3 has not been performed, so the number of STAT3 responsive genes is unknown. Down stream genes that could modify pathfinding are proteins that modulate cytoskeletal dynamics, neurotrophic receptors that would be used for the next leg of the CaP axons ventral journey, or proteins required for ECM interactions. It has been shown in other systems that STAT3 activation prevents apoptosis and this occurs through the transcriptional activation of genes that modulate apoptotic

pathways (Aoki et al., 2003; Brocke-Heidrich et al., 2004; Epling-Burnette et al., 2001a; Epling-Burnette et al., 2001b; Grad et al., 2000; Jee et al., 2002; Liu et al., 2003; Niu et al., 2002; Puthier et al., 1999; Stephanou et al., 2000; Wei et al., 2001). Therefore, in CaP motoneurons STAT3 activation may promote neuronal survival. In zebrafish STAT3 knockdown embryos CaP neural death was not detected by TUNEL up to 3 days after fertilization (data not shown). After 3 days of development STAT3 knockdown embryos die, probably from anterior defects so it is not possible to assess if proper target selection is required for long term CaP survival.

STAT3: Tipping the Balance from Exploratory Branch to Full-Blown Collateral

Formation of an axonal branch requires two processes: branch initiation as well as stabilization. The ectopic branches induced by $STAT3^{AC/NC}$-GFP after RNA injection at the one-cell stage could be due to an increase in both of these processes or only in branch stabilization if extensive exploratory branch formation naturally occurs. Time-lapse video microscopy of pathfinding zebrafish primary motoneurons indicates that the dendrites of these neurons send out numerous exploratory branches that are in a flux of growth and retraction (Jontes et al., 2000). Most retract and are never stabilized. A similar analysis of axonal dynamics has not been performed. However, if the axons of these neurons are as active as the dendrites then there is extensive branch initiating activity.

Stabilization of these exploratory branches would account for the extensive branching seen in embryos expressing constitutively active STAT3.

Late expression of $STAT^{AC/NC}$-GFP from a neurospecific promoter leads to the formation of a dorsal collateral that branches from the axonal trunk of the CaP motoneuron. This collateral branches at the same location as the collateral formed by the MiP motoneuron. Again this ectopic collateral could result from enhanced branch initiation or stabilization of an exploratory branch. The fact that this collateral branches at the same location as the MiP collateral suggests that this is a unique branching position. Perhaps a position just dorsal and caudal to this branch point is an intermediate target, attracting the collateral by a trophic factor. After initially projecting dorsally, most of these ectopic collaterals fail to follow the dorsal pathway. Instead, after a short distance they project caudally through the spinal cord neuropil. Therefore STAT3 enhances trophic responses initiating the collateral or stabilizes an exploratory branch but does not enhance pathfinding along the dorsal pathway.

Time-lapse video microscopy of CaP axonal dynamics is needed to determine if STAT3 initiates or stabilizes branch formation. The CaP neuron may normally explore all pathfinding possibilities including the dorsal pathway. Only those branches will be stably fixed where the sum of transduction signals promotes actin stabilization rather than depolymerization. For growth cone dynamics it is important to think of the combined effect of transduction pathways,

rather than individual pathways. Multiple transduction pathways are activated in growth cones with extensive crosstalk. As an integrated influence, the sum of these pathways either promotes or negates actin assembly. STAT3 or its downstream effectors are clearly only one component among the summed transduction signals within the CaP growth cone. STAT3 may simply "tip the balance" favoring branch formation and pathfinding.

Inactivation of STAT3 and expression of constitutively active STAT3 is not completely penetrant. Trophic factors and the ECM may differ slightly between somites, so that for some CaP neurons STAT3 activation may not be required to promote pathfinding. In morpholino injected embryos, CaP neurons have at most only a 38% chance of pathfinding failure and expression of $STAT3^{AC/NC}$-GFP from the GATA2 promoter only induces an ectopic collateral in 50% of CaP neurons. None of the characterized zebrafish pathfinding mutants are completely penetrant either.

What of STAT3 Knockouts in Mice?

The requirement for STAT3 in CaP pathfinding may be unique to these neurons or to zebrafish. However, the existence of STAT proteins in many organisms and its requirement in numerous cell migration paradigms (see below) suggests that STAT3 may play a role in pathfinding of other neurons and in other organisms. A mouse knockout of STAT3 reveals that at least one STAT3 function is phylogenetically conserved. As mentioned previously STAT3 is required for cell

migrations during gastrulation in zebrafish embryos (Yamashita et al., 2002). STAT3 performs the same function in mice. STAT3 knockout mice show an embryonic lethal phenotype at the onset of cell migrations during gastrulation (Takeda et al., 1997). The uterus rapidly resorbs these embryos shortly after implantation making it impossible in this knockout to assess STAT3 function later in development. To circumvent this early lethality, conditional knockouts have been generated where STAT3 is deleted in specific tissues at later developmental times.

Surprisingly, conditional knockout of STAT3 in motoneurons of the mouse reveals that STAT3 is not obligatorily required for the survival of these neurons during development (Schweizer et al., 2002). Though potential pathfinding defects were not examined. Interestingly these mice do show a motoneuron phenotype though it is not a developmental defect; axotomy of motoneurons in adult mice leads to greater neural death compared to wild-type animals. The authors show that normally the survival factors Reg-2 and Bcl-xl are upregulated in response to lesioning (Schweizer et al., 2002). Both are STAT3 regulated genes (Dusetti et al., 1995; Grad et al., 2000). In the conditional STAT3 knockouts this upregulation is severely reduced accounting for the eventual death of the axotomized neurons.

Another conditional knockout has been generated where STAT3 is deleted in all neurons (Gao et al., 2004). These animals are born at expected Mendelian

ratios, however most fail to thrive and die shortly after birth. The few animals that do survive to adulthood are obese and diabetic. No analysis of neural death, pathfinding, or survival after nerve injury was reported for these animals, so it is impossible to assess from this report if STAT3 plays a role in neural development or repair.

STAT3 in Regenerative vs. Developmental Signal Transduction Pathways

In the mouse motoneuron knockout study (Schweizer et al., 2002) it was postulated that different signaling requirements and pathways are required during neural development vs. regeneration. Other studies have also suggested unique developmental vs. regenerative signaling pathways and STAT3 has been shown to be required for the regenerative/repair process of several neural types [(Liu and Snider, 2001; Snider et al., 2002)]. The requirement for STAT3 in primary CaP pathfinding suggests that these neurons may use a regenerative signaling pathway rather than a standard developmental signaling pathway. Primary motoneurons are only found in fish and frogs; motoneurons of mammals are analogous to the secondary motoneurons of the zebrafish. Interestingly, secondary motoneurons of the zebrafish do not activate STAT3 during pathfinding (data not shown). Zebrafish primary motoneurons may therefore be an excellent system for studying regenerative signaling pathways.

Despite the absence of activated STAT3 in secondary motoneurons, expression of activated $STAT3^{AC/NC}$-GFP under control of the GATA2 promoter in

secondary motoneurons results in an ectopic dorsal collateral identical to that seen in primary CaP motoneurons expressing this construct (data not shown). The ability of STAT3 to promote branching in a neuron that normally does not use activated STAT3 has important implications for the generalized use of STAT3 as a therapeutic target for injured nerves. Perhaps STAT3 activation will promote axogenesis in many neural types.

Neuronal Pathfinding as a form of Cell Migration

Studies of STAT3 function reveal that a central role for this gene in many organisms is the control of cell migration [reviewed in (Hou et al., 2002)]. From a morphological perspective neuronal pathfinding appears quite similar to cell migration. In both cases a portion of the cell membrane protrudes in response to a chemoattractant. The finding that a signal transducer for cell migration is also used for neuronal pathfinding reinforces the cell migration/pathfinding analogy. A central theme therefore emerges whereby a general function of STAT3 is to control cytoskeletal responses to extracellular signals. If STAT3, PI3 kinase, and Rho-GTPase activities are required for both cell migration and neuronal pathfinding, it is likely that other signal transducers known to function in cell migration may be required for neuronal pathfinding as well.

Experimental Procedures

Animals and Embryo Injections

Adult zebrafish of the AB strain and embryos were maintained at 28.5^{0}C. The zebrafish STAT3 gene was amplified by PCR from gastrula stage (8hour p.f.) RNA and cloned into the pSP64T vector upstream from the EGFP gene to create transcription vectors. The STAT3 gene was sequenced to verify there were no

sequence errors. Site directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene product 200518) and mutagenized clones verified by sequencing. One-cell embryos were injected with 0.5 nl of a solution containing RNA or morpholinos in a final concentration of 0.1M KCl and one quarter diluted phenol red solution (Sigma Inc. product P-0290). Capped RNA was synthesized with the Ambion mmessage machine as described by the manufacturer (Ambion Inc. product 1340). Embryos were injected with 250pg of RNA. Morpholinos were purchased from GeneTools LLC (Philomath Oregon). The sequence of the STAT3 morpholino was 5'-CATGTTGACCCCTTAATGTGTCGCT-3'. The sequence of the JAK1 morpholino was 5'-GACACTCGCCAGGCAAAGCTACTTC-3' . Embryos were injected with 2.25ng STAT3 morpholino or JAK1 morpholino. Peptides were synthesized by Anaspec Inc. (San Jose, CA) and dissolved in water at a concentration of 4 mg/ml. They were then diluted into injection buffer as described above and 0.5nl injected into one-cell embryos.

Immunohistochemistry, Cell Transplantations, and Imaging

Embryos were stained with the ZNP1 monoclonal antibody (Antibody Facility, University of Oregon) at a 1:200 dilution or the ZN5 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) at a 1:250 dilution as described by Zeller and Granato (1999). GFP signals were amplified and visualized by staining with anti-GFP rabbit antibody used at a 1:200 dilution (Molecular Probes Inc. product A-6455). FITC conjugated secondary donkey anti-rabbit IgG was obtained from Jackson Immunoresearch (product 711-095-152). Anti-phospho-STAT3 antibody staining (MBL Inc. product D128-3) was performed at a 1:200 dilution as described by (Zeller and Granato, 1999) and visualized with the tyramide-alexafluor 488 kit as described by the manufacturer (Molecular Probes Inc. product T-20912). For dual staining with ZNP1 and antiphospho-STAT3, the ZNP1 staining was visualized with zenon mouse anti-IgG_{2a} alexafluor 546 (Molecular Probes Inc. product Z-25104). Following antibody staining, embryos were equilibrated in 70% glycerol, manually deyolked and mounted between two coverslips on custom manufactured slide holders (Harvard University Biological Laboratories Machine Shop). Cell transplantations were performed exactly as described by Kane and Kishimoto (Kane, 2002). Embryos were imaged with an inverted Leica DMIRB and z-stacks taken with Metamorph Software (Universal Imaging Inc.). Embryos were imaged at low magnification with a Leica MZFLIII dissecting microscope.

Post-Image Processing and Morphometry

Z-stacks were deconvolved using Huygens deconvolution software (Scientific Volume Imaging Inc.) and viewed with Imaris Software (Bitplane AG). Branch lengths were measured using the measurement function found in the Metamorph software suite.

Mosaic GATA2 Promoter Expression

The GATA2 neurospecific promoter was cloned upstream from the 5'UTR sequence of STAT3 transcription vectors and the SV40 early polyadenylation sequence inserted after the GFP sequence. For mosaic expression analysis, linear DNA for injection was obtained by PCR using vector primers flanking the GATA2-STAT3-EGFP insertion. One-cell embryos were injected as described above with 2.5pg DNA of the PCR products.

Acknowledgments

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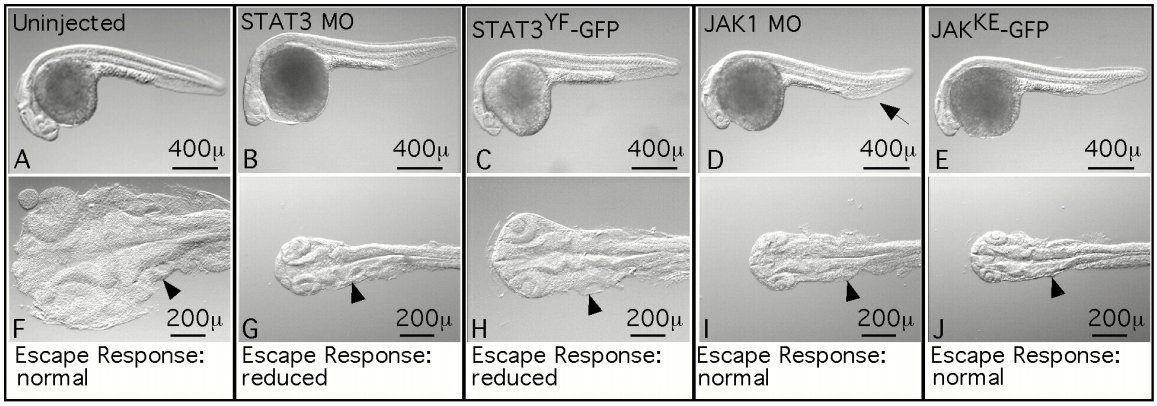

Figure 1. Reduced STAT3 and JAK1 Activity Lead to Similar Morphologies yet Dissimilar Behavior Response

STAT3 and JAK1 activity were reduced by either morpholino injection or expression of a dominant negative version of these proteins. Whole body images are shown in A-E. Note that inactivation of STAT3 or JAK1 lead to identical reduction of anterior structures while the body axis is normal. These deficits are due to reduced cell migration of dorsal mesendoderm during gastrulation. Unlike STAT3 inactivation, JAK1 inactivation also results in a slight spadetail morphology (panel D, arrow). Higher magnification images of the head region (F-J). Arrowheads indicate the anterior limit of the otocyst. STAT3 inactivation eliminates or reduces the escape response such that animals require several proddings before swimming away. In (A) through (E) rostral is to the left and dorsal is up. Images in (F) through (J) are dorsal views with rostral to the left.

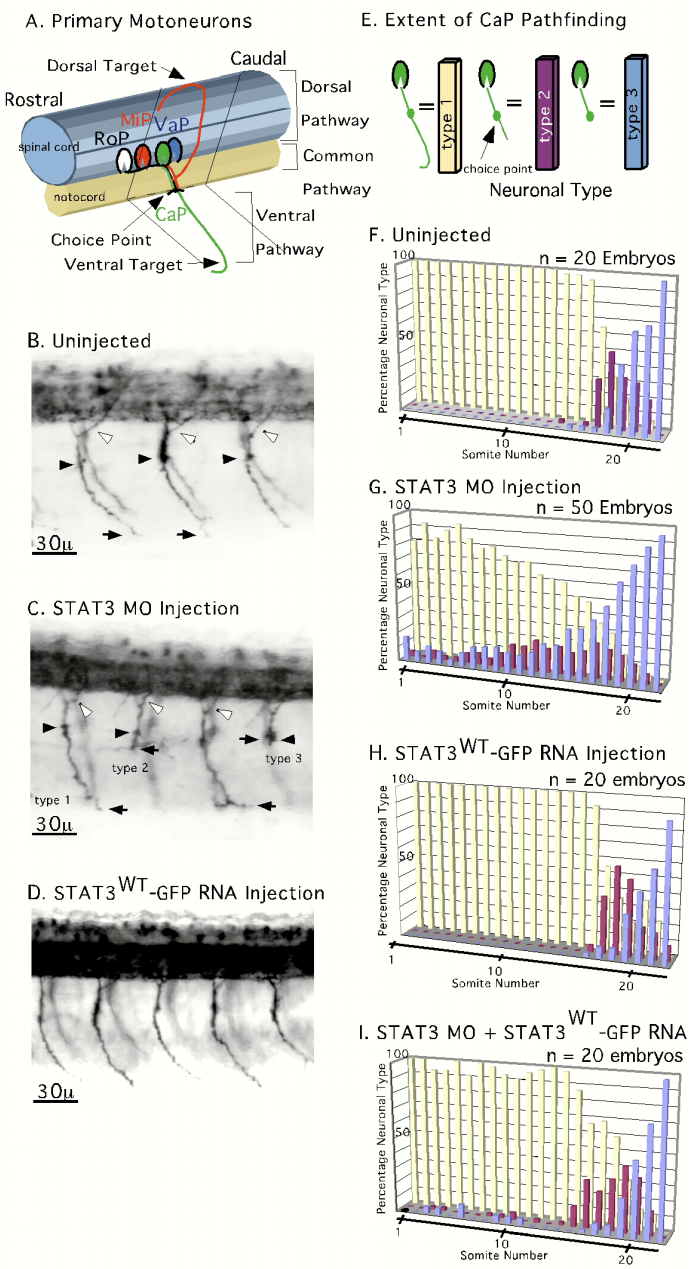

Figure 2. Morpholino Inactivation of STAT3 Results in Pathfinding Defects that can be Rescued by STAT3 RNA Injection

(A) Schematic diagram of primary motoneurons in the zebrafish showing the position relative to the spinal cord. (B-D) ZNP1 staining of primary motoneurons in 27 hour p.f. embryos. (B) Normal pathfinding in uninjected embryos. (C) Pathfinding defects in STAT3 MO injected embryos. (D) Normal pathfinding in embryos injected with RNA encoding a wild type STAT3-GFP fusion. Black arrowheads mark the choice point. Arrows mark the ventral target. White arrowheads point to the dorsal projection of the MiP axon. (E) Schematic diagram of CaP neural types used in quantitations shown in F-I. See text for explanation of quantitations. In images (B) through (D) rostral is to the left and dorsal is up.

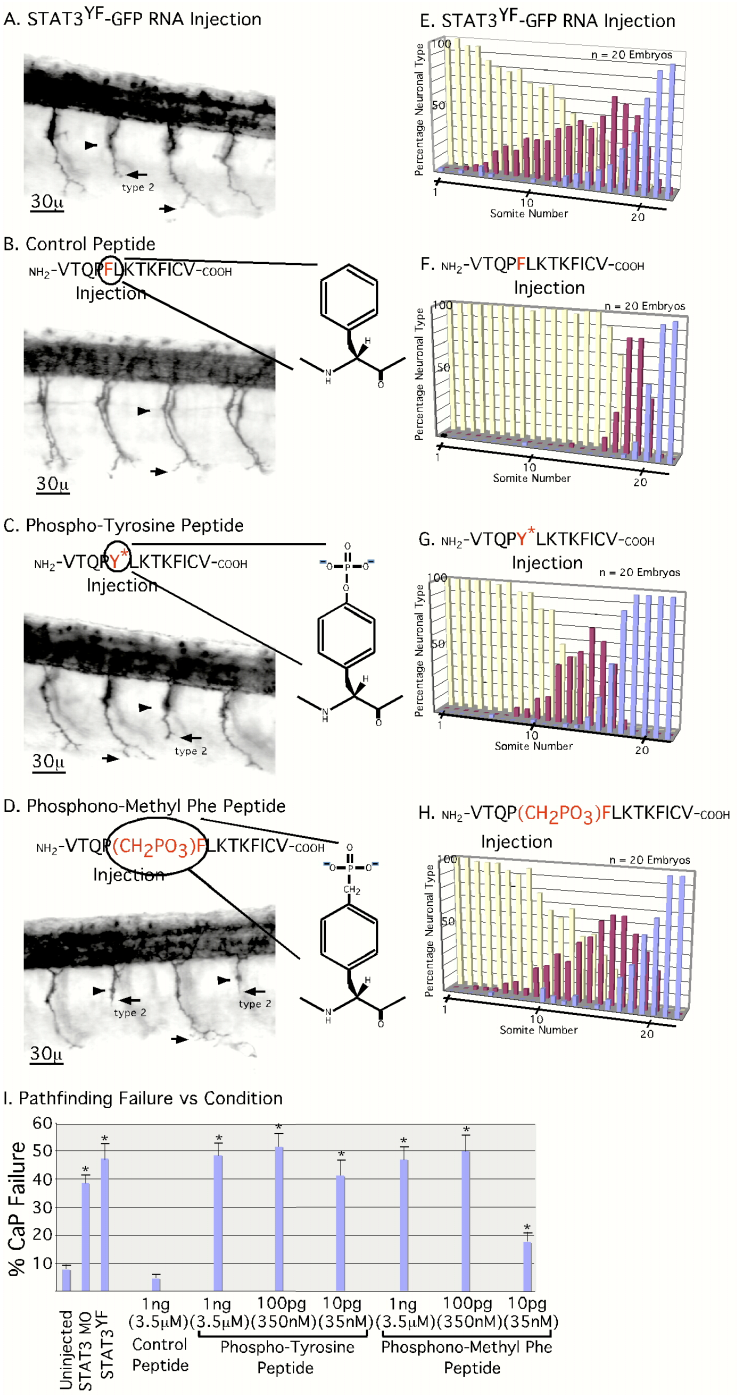

Figure 3. Expression of a Dominant Negative STAT3 or Injection of STAT3 SH2 Domain binding Peptides Results in Pathfinding Failures

(A) Pathfinding defects in embryos injected with RNA encoding a dominant negative STAT3-GFP fusion protein ($STAT3^{YF}$-GFP). The tyrosine in the activation loop of STAT3 that upon phosphorylation binds to the SH2 domain of a STAT3 partner in a STAT3-STAT3 dimer was mutated to phenylalanine. (B) Normal pathfinding in embryos injected with a peptide comprising the activation loop with the activating tyrosine mutated to phenylalanine. (C) Pathfinding defects in embryos injected with a peptide comprising the activation loop and possessing phosphorylated tyrosine. (D) Pathfinding defects in embryos injected with a peptide comprising the activation loop with the activating tyrosine changed to a non-hydrolysable analog of phospho-tyrosine, phosphonomethylene-phenylalanine. (E-H) Quantitation of pathfinding defects relative to somite position in embryos injected as in (A) to (D). Neuronal types used in quantitations are as shown in figure 2 (E). (I) Pathfinding failure as a function of morpholino, RNA, or peptide injection, 20 embryos were examined for each condition. The mean ± standard error of the mean is shown. Bars with asterisks are statistically significant compared to uninjected embryos (t test, $p<0.05$). In images (A) through (D) rostral is to the left and dorsal is up.

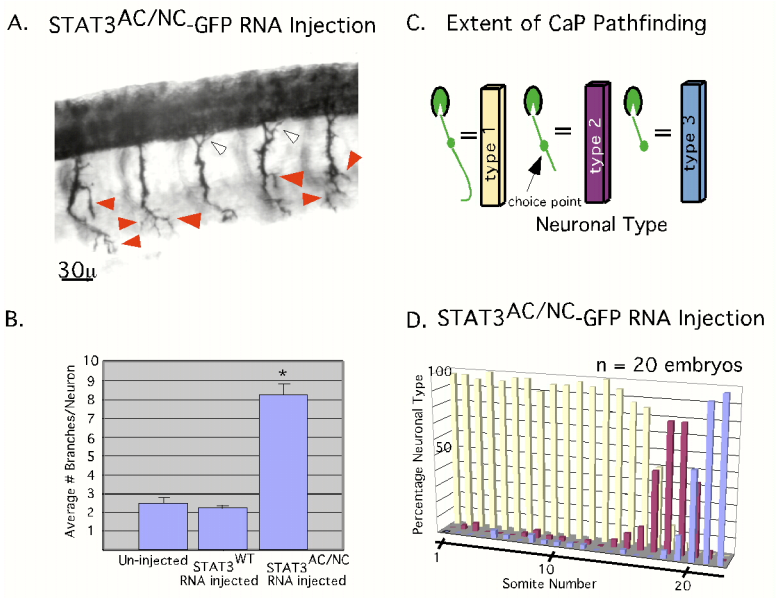

Figure 4. Constitutively Active STAT3 leads to Ectopic Branching
(A) ZNP1 staining of primary motoneurons in 27 hour p.f. embryos injected with RNA encoding a mutated constitutively active version of STAT3 in frame with GFP (STAT3$^{AC/NC}$-GFP). Red arrowheads indicate ectopic branches. White arrowheads mark the dorsal projection of MiP axons. (B) Quantitation of neuronal branches. Bar with asterisk denotes statistically significant increase in the number of branches per CaP neuron compared to un-injected controls or embryos injected with RNA encoding a wild-type STAT3-GFP fusion (t test, $p<0.05$). Neurons of somites 8 to 12 inclusive were counted for 10 individuals yielding 50 neurons per condition. (C) Schematic diagram of CaP neural types used in quantitations shown in (D). (D) Quantitation of CaP pathfinding relative to somite position. Expression of STAT3$^{AC/NC}$-GFP has no affect upon pathfinding success or failure. However as shown in (B), expression of STAT3$^{AC/NC}$-GFP increases the number of branches per CaP axon. In image (A) rostral is to the left and dorsal is up.

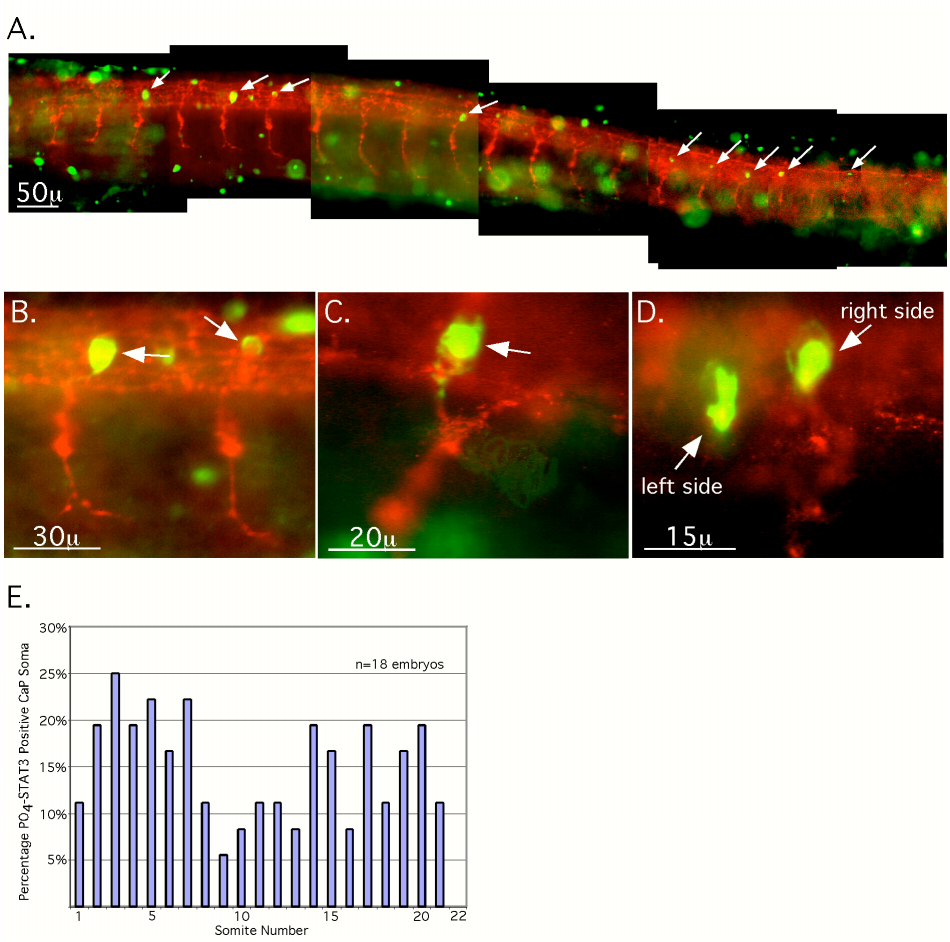

Figure 5. Antibody Staining for Phosphorylated STAT3
(A) Mosaic image of a 24 hour p.f. embryo stained with an antibody to phosphorylated STAT3 (green) and with ZNP1 (red) for primary motoneurons. White arrows mark neuronal cell bodies positive for phosphorylated STAT3. Green cells out of focus are EVL cells. (B-D) Higher magnification images of neuronal soma (white arrows) possessing phosphorylated STAT3. (E) Quantitation of phosphorylated STAT3 positive soma. In (A) through (D) rostral is to the left and dorsal is up.

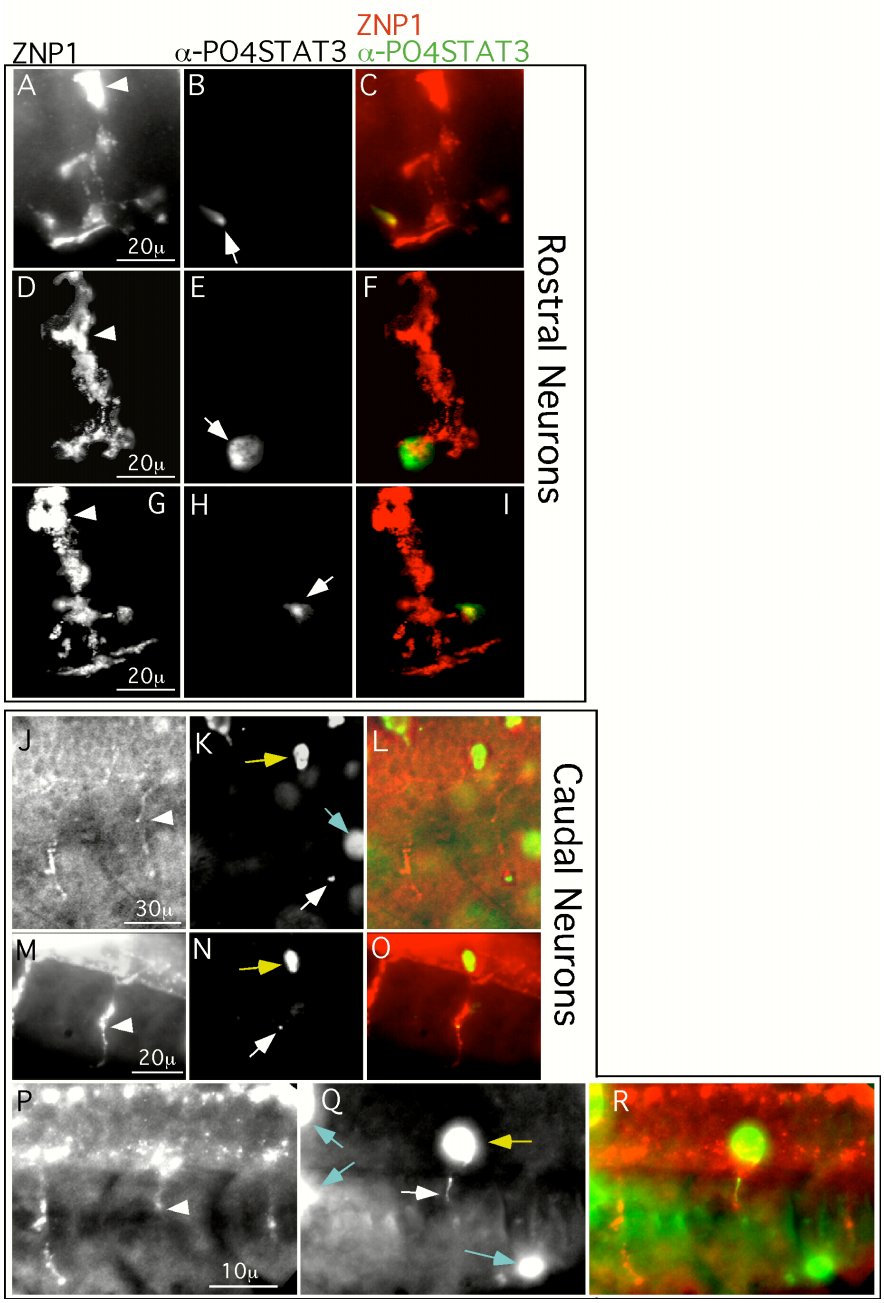

Figure 6. Phosphorylated STAT3 is Found at Growth Cones and along Axons (A-I) Rostral (somites 1 to 7 inclusive) neurons stained for phosphorylated STAT3 or with ZNP1 for primary motoneurons. Phosphorylated STAT3 positive soma are not shown. (J-O) Caudal neurons. (P-R) Caudal neuron having just reached the choice point, showing phosphorylated STAT3 along the axon leading to the phospho-STAT3 positive neuronal soma. White arrowheads mark the choice point, white arrows indicate phosphorylated STAT3 at axonal tips, yellow arrows show phospho-STAT3 positive soma. Blue arrows mark EVL cells possessing phosphorylated STAT3. In all images rostral is to the left and dorsal is up. Images are deconvolved maximal intensity projections of z-stacks.

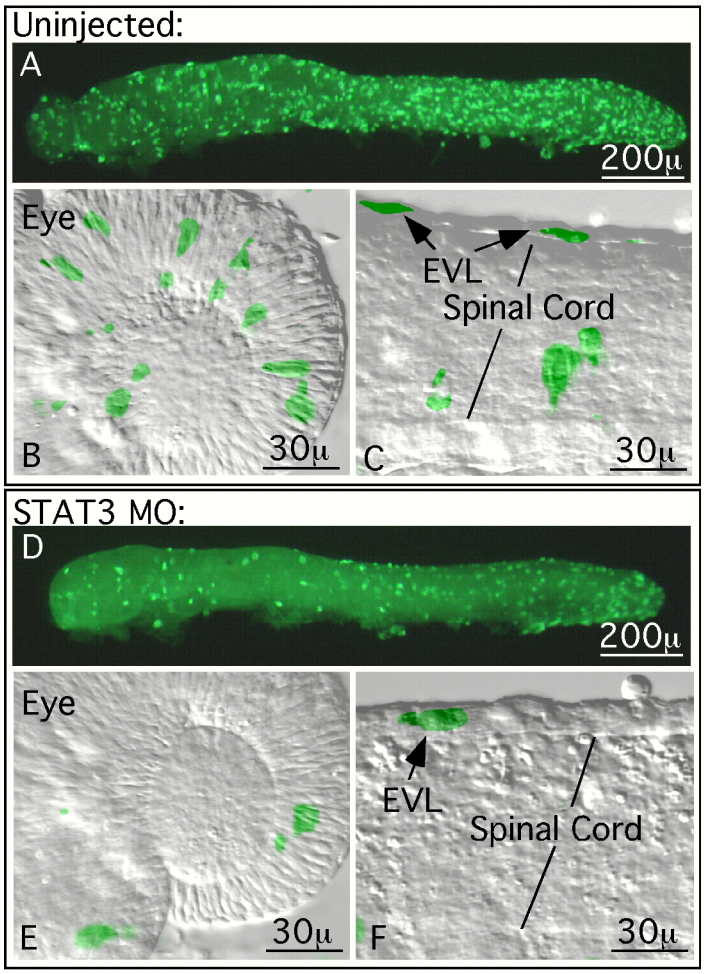

Figure 7. Morpholino Injection reduces STAT3 signaling
(A-C) Uninjected embryos at 24 hour p.f. stained for phosphorylated STAT3 (A, green fluorescence) and viewed at higher magnification with fluorescence and DIC (B and C). (D-F) STAT3 morpholino injected embryos stained and viewed as in (A) to (C). In all images rostral is to the left and dorsal is up.

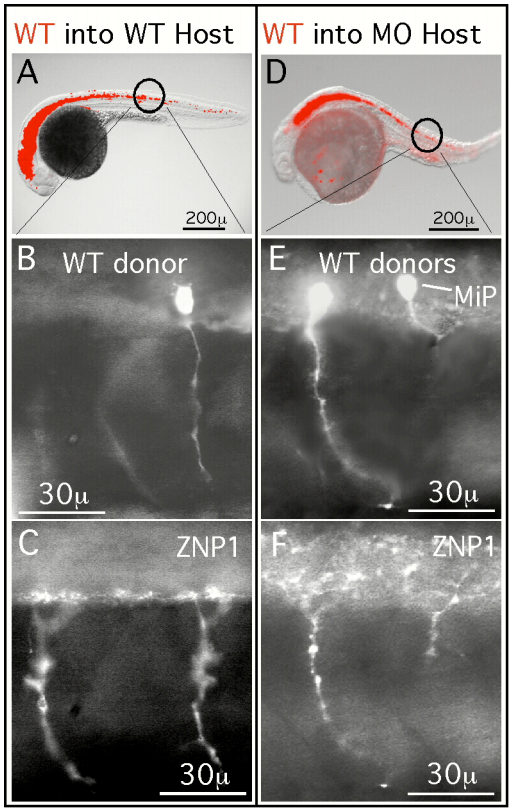

Figure 8. Cell transplantations show a Cell Autonomous Requirement for STAT3 in Pathfinding
(A-C) Control transplantations where wild-type blastomeres, red cells in (A), were transplanted into wild-type hosts. (D-F) Transplantations of wild-type blastomeres, red cells in (D), into STAT3 morpholino injected hosts. In all images rostral is to the left and dorsal is up.

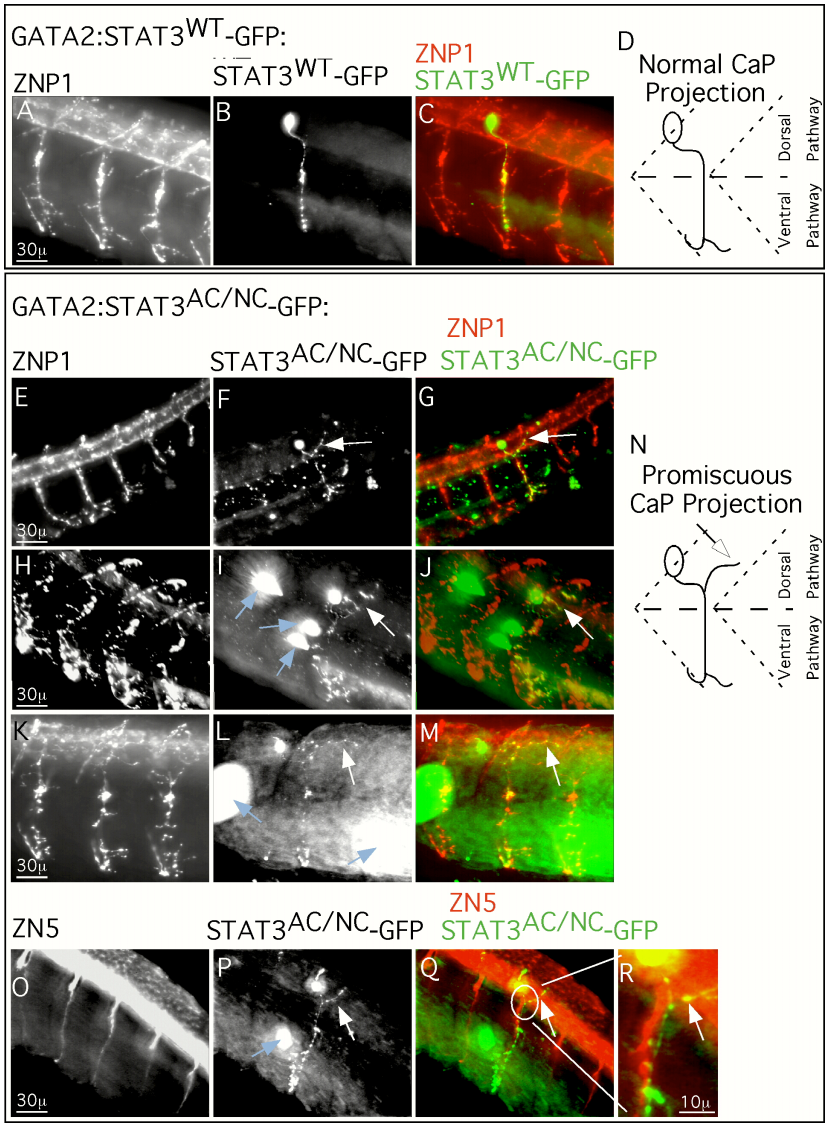

Figure 9. Late Expression of Constitutively Active STAT3 leads to Promiscuous Pathfinding

(A-C) A wild-type STAT3-GFP fusion protein was expressed under the control of the GATA2 neuro-enhancer (GATA2:STAT3^{WT}-GFP). Expression from this promoter begins at 24 hours p.f. and the embryos shown are at 48 hours p.f. (D) Diagram of the normal CaP projection. (E-M) Neurons of 48 hour p.f. embryos expressing constitutively active STAT3$^{AC/NC}$-GFP under the control of the GATA2 neuro-enhancer. White arrows mark the ectopic dorsal collateral resulting from this expression. Blue arrows mark out of focus EVL cells that occasionally show expression from this promoter construct. (N) Diagram of aberrant CaP pathfinding indicating the position of the ectopic dorsal collateral. (O-R) Embryos expressing the constitutively active STAT3$^{AC/NC}$-GFP fusion protein stained with the ZN5 antibody that recognizes secondary motoneurons. Panel R is a magnified view of the circled region in panel Q. In all images rostral is to the left and dorsal is up. Images are deconvolved maximal intensity projections of z-stacks.

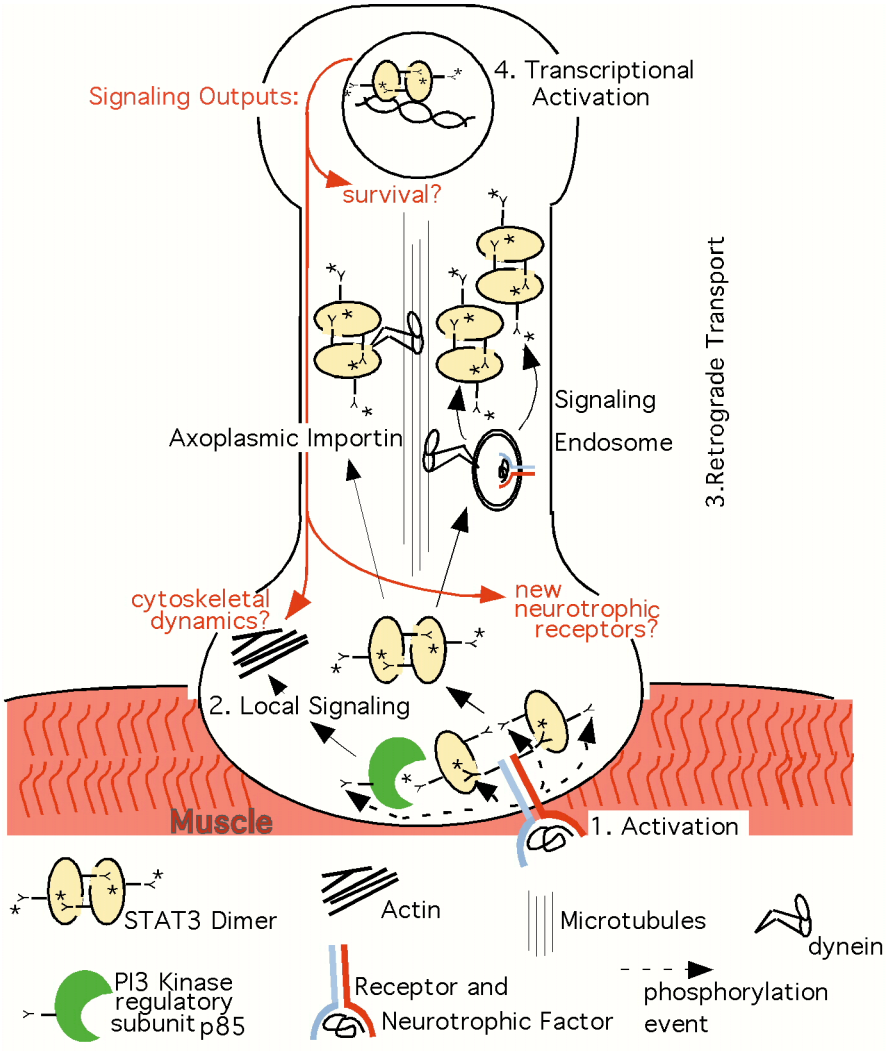

Figure 10. Model for Local and Retrograde STAT3 Signaling Required for Neuronal Pathfinding

STAT3 activation occurs at or just after the CaP neuron reaches the choice point. A neurotrophic factor at the medial muscle surface leads to receptor dimerization (Activation) followed by phosphorylation events that produce STAT3 dimers and activation of the PI3 kinase regulatory subunit (p85α). Phosphorylated p85α promotes actin polymerization through activation of downstream transduction intermediates (Local Signaling). STAT3 signals are translocated along the axon by signaling endosomes that continually produce STAT3 dimers as they travel (Signaling Endosome) or STAT3 dimers formed at the growth cone are bound by axoplasmic importins and are transported by dynein (Axoplasmic Importin). Once in the nucleus STAT3 dimers activate transcription of target genes (Transcriptional Activation) producing signal outputs (Signaling Outputs) that may promote survival, control cytoskeletal dynamics, or provide receptors for the neurotrophic factor produced from the next intermediate target along the CaP neurons path.